The new levels of redox regulation of S-adenosylmethionine synthesis

Los nuevos niveles de regulación redox de la síntesis de S-adenosilmetionina

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KEYWORDS

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PALABRAS CLAVE

S-adenosilmetionina; glutatión; estrés oxidativo; residuos de cisteína; óxido nítrico; enfermedades hepáticas; regulación redox; daño hepático agudo.

SUMMARY

S-adenosylmethionine is a very versatile compound known to be involved in as many reactions as ATP. Its role as methyl donor is key for the production of a large variety of molecules, as well as, for the modification of proteins and nucleic acids. Therefore, it is not surprising that impairments in the methionine cycle are found in many diseases including liver pathologies, Alzheimer or rare diseases. In most of these cases, reductions in S-adenosylmethionine concentrations correlate with the presence of oxidative stress. This fact prompted the study of a putative redox regulation of the methionine cycle that has been focused especially on methionine adenosyltransferases, the enzymes that synthesize S-adenosylmethionine. This review is intended to provide an outline of the new levels at which the redox control of these enzymes is exerted and their importance for liver pathology, a field in which we have made several key contributions.

RESUMEN

La S-adenosilmetionina es un compuesto muy versátil, conocido por participar en casi tantas reacciones como el ATP. Su papel como donante de grupos metilo es esencial para la producción de una gran variedad de moléculas, así como para la modificación de proteínas y ácidos nucleicos. Por ello, no resulta sorprendente que se hayan detectado alteraciones en el ciclo de la metionina en una gran variedad de patologías, que incluyen desde enfermedades hepáticas hasta el Alzheimer o enfermedades raras. En muchos de estos casos la reducción de los niveles de S-adenosilmetionina se acompaña de la presencia de estrés oxidativo. Este hecho ha inducido el estudio de una posible regulación redox del ciclo de la metionina, que se ha enfocado principalmente a las metionina adenosiltransferasas, que son las enzimas encargadas de la síntesis de S-adenosilmetionina. Esta revisión pretende dar una visión global de los nuevos niveles a los que se ejerce el control redox de estas enzimas y su importancia en hepatopatología, campo en el cual hemos realizado importantes aportaciones.

1. INTRODUCTION

Nature has evolved to create several essential compounds with high versatility, so that a few molecules can be used in a large variety of reactions. Initially, this represents an evolutionary advantage reducing the number of enzymes and compounds required for life. However, this same aspect imposes a serious drawback, since alterations affecting these few processes may lead to disease. Among these versatile compounds two have been shown of special relevance for the cell, ATP and S-adenosylmethionine (SAM). This last metabolite was described for the first time in 1951 by Cantoni as an "active methionine" able to transmethylate in the absence of ATP (1). Although at that moment its exact nature was unknown, in this same paper the author indicated that SAM was a product of the reaction between ATP and methionine. Finally, in 1953 this researcher described the reaction catalyzed by the "methionine activating enzyme" and the chemical nature of SAM (2).

The number and variety of biological processes that involve the use of SAM has increased along the years, so that nowadays the catalogue of reactions using this metabolite is as large as that of the processes utilizing ATP (3). This versatility of SAM relies in its chemical structure, which contains a quiral sulfonium group through which carbon-sulfur bonds with aminopropyl, methyl and adenosine groups are established. These groups are donated to a large variety of substrates upon nucleophilic attack of the corresponding carbon-sulfur bond (reviewed in (4,5)). Additionally, SAM can also transfer the rybosyl or amino groups in processes that have been described in bacteria or plants (3,6,7).

Methyltransferases consume the majority of the SAM produced in any organism in reactions leading to the synthesis of small molecules (i.e. neurotransmitters, phosphatidylcholine, etc.), the modification of proteins (i.e. histones) and DNA, among

others. The remaining SAM is involved in the synthesis of 5'-halogenated derivatives in marine organisms (adenosine group), queuosine (rybosyl group) and other hypermodified nucleosides (3-amino carboxypropyl group), pelargonic acid (amino group), polyamines, molecules involved in bacterial signaling and ethylene (aminopropyl group). SAM can also be used both as cofactor or substrate by SAM radical proteins, which catalyze probably the last set of reactions involving this compound that has been identified to date (3,8). In mammals, transmethylations consume approximately 95% of the SAM produced and the rest is used for SAM decarboxylation, being decarboxylated SAM the genuine aminopropyl donor for the synthesis of the polyamines spermidine and spermine (4,9).

2. THE METHIONINE CYCLE IN MAMMALS

SAM synthesis is carried out by methionine adenosyltransferases (MATs; EC 2.5.1.6), also known as SAM synthetases, this step being the first and rate limiting of the methionine cycle (4) (Figure 1). Most of the information regarding this pathway has been obtained in liver, where a human adult processes up to 48% of the ingested methionine (9-11), but slowly information concerning other organs is becoming available (i.e. the cochlea (12)). The need for increasing the existing knowledge in extrahepatic tissues has been emphasized when impairments of the methionine cycle, and especially reductions in SAM concentrations or hyperhomocysteinemia, have been associated with different pathologies. Most of the diseases in which alterations of the pathway have been demonstrated concern the liver (i.e. hepatitis, cirrhosis, acute liver failure, hepatocellular carcinoma) (9,13), but its impairment in cardiovascular diseases (14,15), Alzheimer (16-18), psoriasis (19), rare diseases (i.e. Wilson disease) (20-22) and even hearing loss (12,23) has been also shown. It is not surprising that such a

variety of pathological states concur with an anomalous function of the methionine cycle, given not only the large diversity of biological processes involving SAM, but also the connections of this pathway with additional key routes and its dependence on the nutrient status, as will be explained below.

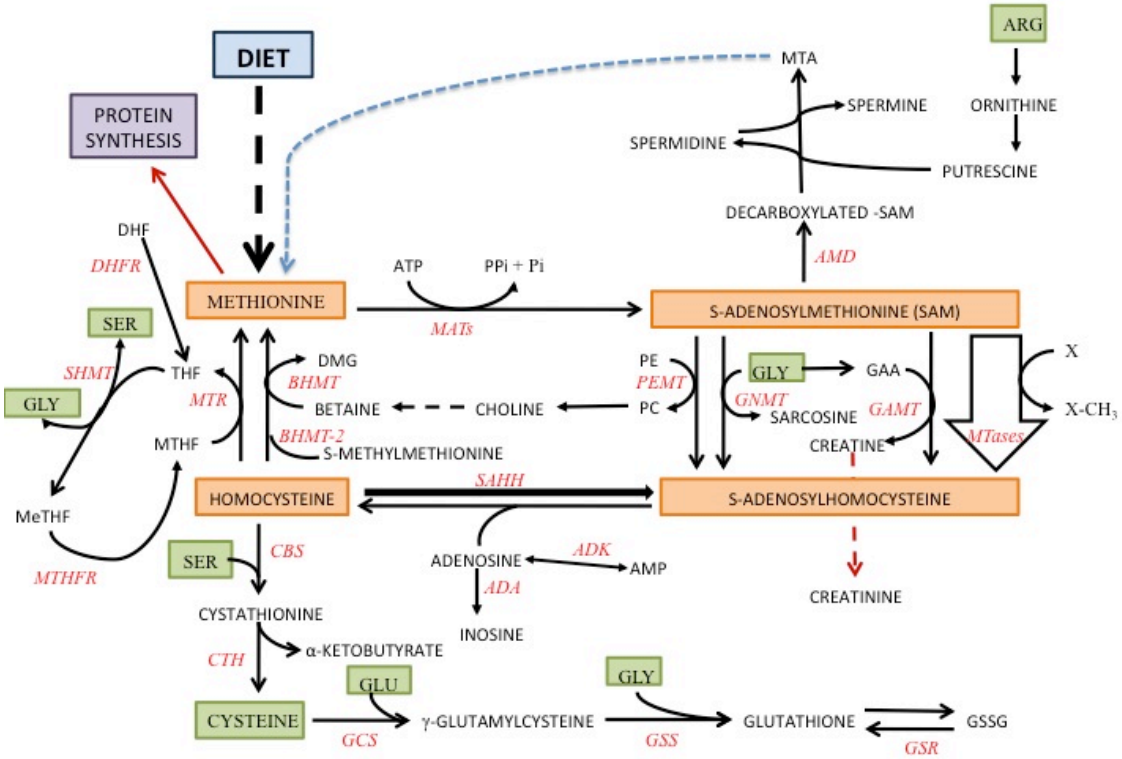

Figure 1. Mammalian methionine cycle and associated pathways. The scheme shows the main reactions involved in the methionine cycle, polyamine synthesis, folate cycle, trans-sulfuration and glutathione synthesis. A discontinuous blue line indicates the methionine salvage pathway. Enzymes and metabolites appear abbreviated as follows: MATs, methionine adenosyltransferases; PEMT, phosphatidylethanolamine N-methyltransferase; GNMT, glycine N-methyltransferase; GAMT, guanidinoacetate N-methyltransferase; MTases, methyltransferases; SAHH, S-adenosylhomocysteine hydrolase; ADK, adenosine kinase; ADA, adenosine deaminase; CBS, cystathionine β-synthase; CTH, cystathionase; GCS, γ-glutamylcysteine synthetase; GSS, glutathione synthetase; GSR, glutathione reductase; BHMT, betaine homocysteine methyltransferase; BHMT2, betaine homocysteine methyltransferase 2; MTR, methionine synthase; DHFR, dihydrofolate reductase; SHMT, serine hydroxymethyltransferase; MTHFR, methylene tetrahydrofolate reductase; AMD, S-adenosylmethionine decarboxylase; THF, tetrahydrofolate; DHF, dihydrofolate; MTHF, 5-methyl tetrahydrofolate; MeTHF, methylene tetrahydrofolate; MTA, methylthioadenosine; GSSG, glutathione oxidized form; GAA, guanidinoacetate; X, any methyl acceptor.

Methionine was discovered in 1923 by Mueller (24), and found to be essential for mammals in 1937, when growth retardation was detected in rats fed methionine-free diets (25). Hence, this amino acid must be obtained from the diet, and after absorption,

needs to be distributed to the whole organism, where requirements of each organ are not equal, as mentioned previously. Once into the cell the use of this amino acid is shared between protein synthesis and the methionine cycle, and part of the methionine consumed in these pathways can be recycled. Mammalian cells can recover part of the methionine used in the methionine cycle by homocysteine (Hcy) remethylation and also by the methionine salvage pathway, which has a minor role. Hcy remethylation requires additional methyl donors to synthesize methionine, being those mainly 5-methyl tetrahydrofolate (MTHF), but also betaine and S-methylmethionine (vitamin U). These remethylation reactions are catalyzed by a vitamin B12-dependent methionine synthase (MTR; EC 2.1.1.13), betaine homocysteine methyltransferase (BHMT; EC 2.1.1.5) and BHMT2 (EC 2.1.1.10), respectively (26,27). The Hcy required for this purpose is generated as an intermediate in the trans-sulfuration pathway that converts methionine into cysteine, and in which SAM synthesis by MATs is the first step. Transmethylations then use the methyl donor rendering the methylated products together with demethylated SAM, named S-adenosylhomocysteine (SAH). Three methyltransferases in charged of the synthesis of small compounds are the main consumers of hepatic SAM. Namely, glycine N-methyltransferase (GNMT; EC 2.1.1.20), phosphatidylethanolamine N-methyltransferase (PEMT; EC 2.1.1.17) and guanidinoacetate N-methyltransferase (GANMT; EC 2.1.1.2) which synthesize sarcosine, phosphatidylcholine and creatine, respectively (13).

The byproduct of transmethylations, SAH, is a potent inhibitor of most methyltransferases and its hydrolysis is carried out by SAH hydrolase (SAHH or AHCY; EC 3.3.1.1) in the only reversible reaction of the methionine cycle, which renders adenosine and Hcy (9). Elimination of SAH is key in order to maintain an appropriate methylation level that depends on the SAM/SAH ratio, also known as the

methylation index. Calculations carried out in several tissues have established that the optimal hepatic ratio is approximately 4, although this number is higher for other tissues such as the lung, where the preferred methylation index is around 9 (28). Preservation of these optimal SAM/SAH ratios depends on an efficient removal of Hcy, adenosine or both.

Hcy levels depend on its catabolism through the remaining reactions of the trans-sulfuration pathway leading to cystathionine and cysteine, its elimination into the blood or, when methionine levels become low, its remethylation. The metabolic branch point represented by Hcy favors its use for cysteine synthesis through the consecutive activities of cystathione β-synthase (CBS; EC 4.2.1.22) and cystathionase (CTH; EC 4.4.1.1). These two enzymes exhibit higher Km values for Hcy than remethylating proteins, are activated by high levels of SAM and require vitamin B6 (26,29,30). In parallel, adenosine can be utilized by adenosine deaminase (ADA; EC 3.5.4.4) or adenosine kinase (ADK; EC 2.7.1.20) to produce inosine or AMP, respectively (26). All these reactions connect the methionine cycle with multiple pathways in which regulators of the former are generated, hence creating a series of feedback loops whose complexity is not totally understood. Moreover, all the reactions described above do not show identical importance in every tissue, due to differences in gene expression patterns, and hence, on the enzymes or isoenzymes encountered in each cell type.

Main players in the regulation of the hepatic methionine cycle are metabolites, from the same or related pathways, hormones and nutrients, some of their key effects being summarized below. Among the metabolites, SAM deserves a special consideration, since its role includes the activation or inhibition of several enzymes such as MATs, CBS and CTH, as well as the inhibition of BHMT expression (29,31,32). SAM further contributes to the inter-regulation between pathways by inhibiting

methylene tetrahydrofolate reductase (MTHFR; EC 1.5.1.20) of the folate cycle and regulating AMP kinase (AMPK; EC 2.7.11.31) phosphorylation (33,34). Nutrients such as vitamins of the B group, or their derivatives, also have a relevant role as cofactors or substrates for MTR, CBS and CTH, but also as inhibitors of GNMT in the form of MTHF (35-38). Moreover, methionine levels have been shown to regulate expression of MAT genes (39), and hormones seem to exert their role preferentially at the expression level, as occurs with glucocorticoids and MAT genes (40). Redox regulation is exerted by glutathione, and the ratio of its reduced (GSH) and oxidized (GSSG) forms (41-43), nitric oxide (44), hydroxyl radicals (45), $NADP^+$ (46), copper (20,47), etc. on MATs and SAHH. Additionally, oxidative stress inhibits MTR and activates CBS through changes in the redox status of their cofactors, vitamin B12 and the heme group, respectively (48).

3. METHIONINE ADENOSYLTRANSFERASES

MATs are a family of enzymes highly conserved between Bacteria and Eukaryota. This conservation expands from the amino acid sequence to the protein structure, facts that allowed us to propose MATs as a good phylogenetic marker back in 2004 (49). Moreover, MATs from Archaea despite their low sequence conservation (~18%) still preserve the amino acid residues involved in catalysis (50), suggesting that the mechanism for SAM synthesis has been maintained throughout evolution (5). This family of enzymes uses methionine and ATP as substrates in a reaction that requires Mg^{2+} and K^+ ions and which takes place in two steps: 1) the transfer of the adenosyl moiety of ATP to the sulfur atom of methionine, generating SAM and triphosphate; and 2) the hydrolysis of the triphosphate originating pyrophosphate and inorganic phosphate and allowing SAM liberation from the active site (4,5).

There are three MAT genes in mammals, named *MAT1A*, *MAT2A* and *MAT2B,* which encode two types of catalytic subunits, MATα1 and MATα2, and the regulatory subunit MATβ, respectively (Figure 2). The high level of identity exhibited by MATα1 and MATα2 (85% at the amino acid level), is not shared by MATβ, which is a non-related protein of the PFAM 04321 oxidoreductase family (4,5). Four splicing forms of MATβ have been detected in hepatoma cells, from which the V1 form is the protein encountered in normal tissues and which has been evaluated in most studies published to date (51).

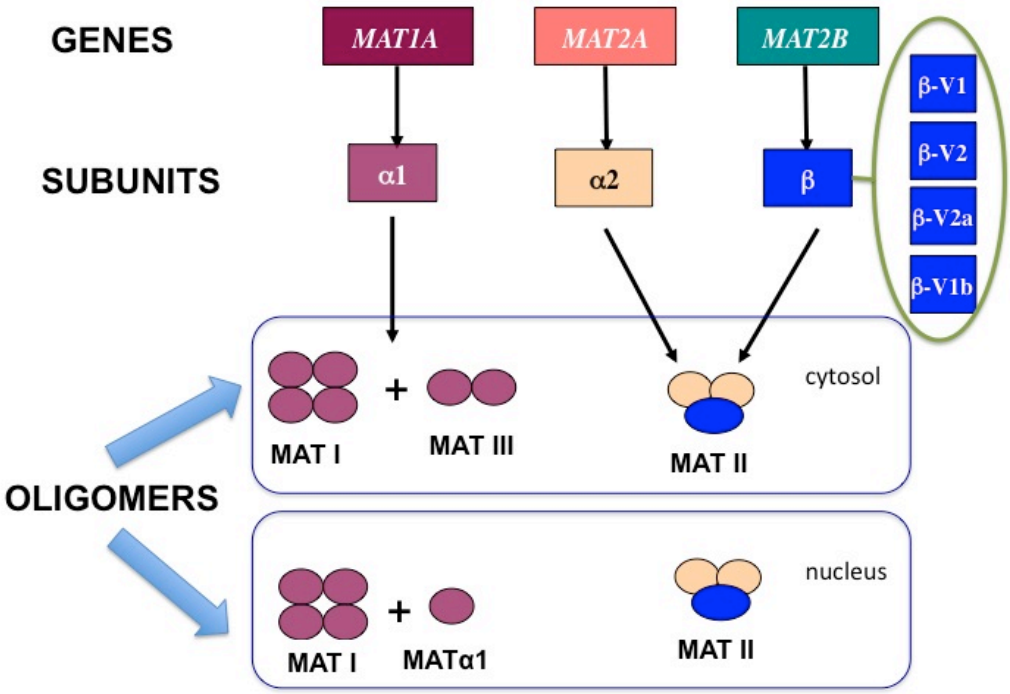

Figure 2. Methionine adenosyltransferase nomenclature. The scheme summarizes the nomenclature used in the field regarding genes, subunits, splicing forms (circled in green) and the oligomeric association of the subunits to render the isoenzymes found in the cytosol and the nucleus.

MAT subunits associate into the three isoenzymes that have been detected in mammalian cells, named MAT I, MAT II and MAT III (Figure 2). Their characterization was carried out after their isolation from liver (MAT I and III) or kidney (MAT II) and found to be homo- or hetero-oligomers (4). Precisely, MAT I and

MAT III were identified as homo-tetramers and homo-dimers of MATα1 subunits (52-54), respectively, whereas MAT II was classified as a hetero-oligomer of MATα2 and MATβ subunits (55,56). The affinity for methionine of the three isoenzymes expands the whole micromolar range (reviewed in (4,5)), some differences being encounter between laboratories, but mean data corresponding to the following values: 3 μM for MAT II; 30 μM for MATα2 homo-oligomers; 100 μM for MAT I; and 1 mM for MAT III. In contrast, V_{max} values showed the opposite trend, MAT II < MATα2 homo-oligomers < MAT I < MAT III. These kinetic parameters determine the capacity for SAM synthesis of each cell type, under normal or pathological conditions, according to the isoenzyme expressed and the level attained.

The classical knowledge on MATs by the 90s can be summarized as follows (reviewed in (4)): i) normal liver was the solely tissue where *MAT1A* was expressed; ii) *MAT2A* was expressed in extrahepatic tissues, hepatopathologies and fetal liver; iii) *MAT2B* expression followed that of *MAT2A,* although at lower levels; iv) MAT isoenzymes were cytosolic proteins; v) MAT II was probably a heterotetramer ($\alpha2_2\beta_2$); v) binding of MATβ to MATα2 increased the affinity of the latter for methionine; vi) SAM inhibits MAT I and II isoenzymes, but is an activator of MAT III; vii) MAT III is activated by dimethylsulfoxide; viii) cysteine residues were found to be important for MAT I/III activity and in order to maintain the association state; and ix) SAM was transported from the cytoplasm to other subcellular compartments as required. During my posdoctoral in Mato's group in the early 90s we were able to provide additional evidences concerning MAT I and MAT III (reviewed in (4). Those included results obtained both *in vitro* and *in vivo*, the main achievements being as follows: i) further insights into the role of the cysteine residues; ii) the modulation of the activity by glutathione levels; iii) regulation of the isoenzyme activity by PKC phosphorylation; iv)

the identification of the putative ATP binding site by photoaffinity labeling; v) the regulation by glucocorticoids; and vi) the differential expression pattern during liver development. Based on these accumulated knowledge, several questions remained unanswered, being their response the objective of our group in the last 20 years.

4. OUR CONTRIBUTION TO UNDERSTAND MAMMALIAN METHIONINE ADENOSYLTRANSFERASES: FROM STRUCTURE TO NEW REGULATORY LEVELS.

The objective since we started our independent work by 1994 was to understand several aspects of MAT regulation that were poorly addressed, despite the intensive work of several groups since their discovery. Some of the limiting facts for the field at that time related to the difficult purification procedures to obtain the isoenzymes and the lack of good antibodies or structural information. Therefore, our initial work was focused on these aspects to develop better protocols and tools that allowed progress in the study of MATs, and which latter favored additional studies on animal models, as will be explained below.

4.1 Association of α-subunits is required to obtain active MATs.

The first objective of our work was to understand the need for oligomerization of MAT α-subunits, and for this purpose we undertook the crystallization of rat MAT I and MAT III. Our initial attempts were based in the use of the rat liver purified proteins, but the heterogeneity of the sample precluded crystallization. Next, we tried to use the recombinant protein overexpressed in *E. coli* (53,54), and several important problems had to be faced: 1) the level of soluble protein was very low; 2) it was impossible to separate the recombinant protein from the bacterial MAT due to their high homology and identical chromatographic behavior; and 3) refolding of MAT I/III from the

inclusion bodies required a protocol that had to be established. Nevertheless, we decided to pursuit the design of a specific refolding protocol (57), rendering large amounts of soluble protein, and that latter revealed its use for additional purposes. The success of this refolding protocol relied on the use of very low protein concentrations, the addition of Mg^{2+} to the buffers, the utilization of two refolding steps (fast and slow), and the maintenance of the 10 cysteines of rat MATα1 in a reduced state during the whole procedure (57). Characterization of the refolded protein showed that both MAT I and MAT III isoenzymes could be obtained, and their interconversion was possible just by concentration or dilution of the sample (57). Moreover, kinetic parameters and circular dichroism spectra of the refolded proteins were similar to those of the isoenzymes purified from rat liver (57,58). These facts, together with the large amount of refolded and purified MAT I/III obtained, led us to use these proteins in new crystallization attempts that were successful.

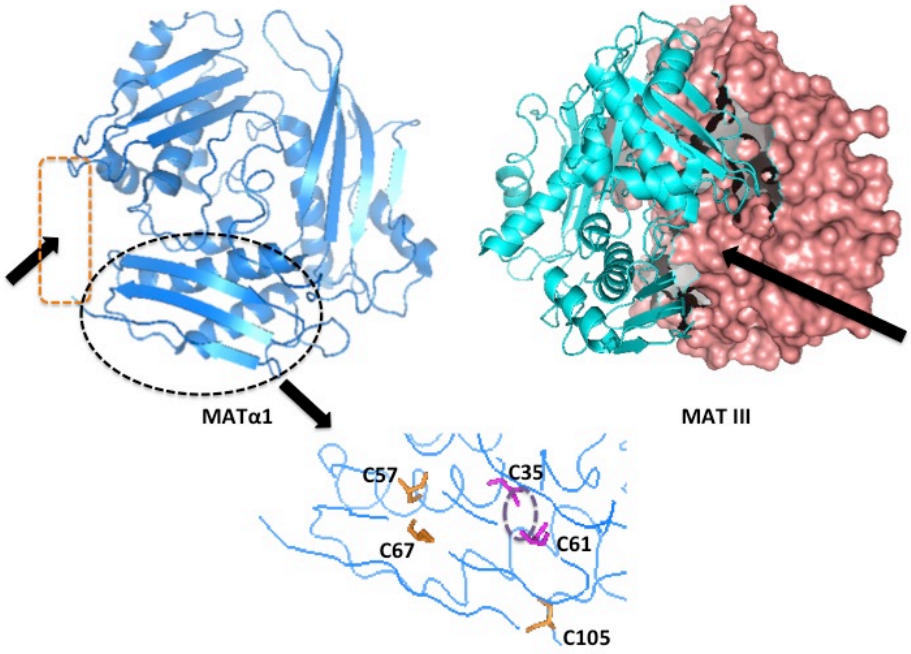

Figure 3. Structural details of MATα1 and its dimer. A cartoon representation of the secondary elements found in rat MATα1 monomers is shown in the upper left panel. An arrow indicates the position of the flexible loop of access to the active site in the completely folded subunit and the central domain is highlighted. The crystal structure of the MAT III appears on the right side of the figure, an arrow indicating the entrance to the active site located at the dimer interface. The lower part of the figure shows a magnification of the central domain,

including the five cysteine residues located in this area (C35, C61, C57, C67, C105). The position of the intrasubunit disulfide (C35-C61) appears indicated by a circle.

The crystal structure of rat MAT I was reported in 2000, becoming the first mammalian structure solved for this family of proteins (59). MATα1 monomers showed a three-domain organization similar to that previously described for the *E. coli* counterpart (5) (Figure 3). The domains were formed by nonconsecutive stretches of the protein chain, each of them containing a β-sheet (59). The three sheets made up a large hydrophobic surface that constituted the interaction interface between monomers in the dimer. The two active sites of each dimer located at this interface, opposite one to another, and their structure required residues of both subunits for catalysis (59,60). This organization therefore explained the need for association exhibited by MATα1 subunits, and the fact that dimers were the minimum active isoenzymes. Production of the crystals in the presence of substrates or certain analogues also allowed the identification of key residues for catalysis (59,60). Precisely, F251 was identified as key for methionine binding (59), whereas the role in ATP binding of a P-loop previously identified by photoaffinity labeling was confirmed (60,61). The next level of association involved the central domains of each monomer, which provided the few residues that allowed tetramerization, being the resulting interaction pattern much more limited then that previously described for *E. coli* MAT (62). How these interactions contribute to the changes in affinity and V_{max} shown between MAT I and MAT III is still unknown and requires further investigation.

4.2 Interconversion between MAT I and III isoenzymes is blocked by an intrasubunit disulfide bond.

The next question we addressed concerned the interconversion between MAT I and MAT III, and specifically what blocks this exchange, which is not observed in the

liver purified isoenzymes. The sequence of MATα1 includes 10 cysteine residues and N-ethylmaleimide modification of just one of them was responsible for a large loss of enzymatic activity. Moreover, modification of an additional sulfhydryl group led to inactive dimers (63). Analysis of mutants generated in all the cysteines confirmed the role of these residues in activity, and demonstrated that those comprised between C35 and C105 were involved in the control of the MAT III/I isoenzyme ratio (64). Latter on, C121 was identified as the target for inactivation by nitric oxide and hydroxylation (44,45,65), a role explained by its location in the loop of access to the active site (59,60). However, our initial studies using chemical modification also revealed that in the isoenzymes purified from rat liver two cysteine residues remained elusive, and that only after reduction they became accessible to N-ethylmaleimide modification (63,66). These data suggested the presence of a disulfide bond within MAT I and MAT III, which was identified as an intrasubunit bond involving C35 and C61 in the isoenzymes isolated form rat liver (66) (Figure 3). The crystal structure of MAT I, obtained under reducing conditions, showed that these residues were well oriented and at a distance short enough to form such an intrasubunit bond (59).

The role of the disulfide was further explored taking advantage of the available refolding procedure and cysteine mutants prepared on MATα1 (62). For this purpose, refolding was carried out under reduced (with DTT) or mild oxidative conditions (with GSH/GSSG mixtures). Analysis of the resulting refolded isoenzymes showed that interconversion between MAT I and MAT III could be produced only when the proteins were fully reduced (62). Moreover, mass spectrometry evaluation of the refolded proteins revealed that this interconversion was blocked by the presence of the C35-C61 disulfide bond in the isoenzymes resulting from refolding under mild oxidative conditions (62). Once more, the use of the available structural data allowed us to

propose that the role of this intrasubunit disulfide relies in its ability to stabilize the β-sheet of the central domain involved in dimer-dimer interactions. Therefore, production of the disulfide at different steps during folding would result in stable tetramers or dimers.

4.3 Unfolding of MAT I and MAT III takes place through an intermediate state.

Our two-step refolding protocol suggested the requirement of an intermediate state that must be well populated to obtain a correct association pattern of the MATα1 monomers into dimers, in order to avoid aggregation (57). Unfolding studies carried out on MAT III using urea as denaturant confirmed this hypothesis, and demonstrated a three-state mechanism that is reversible (58). The intermediate identified by gel filtration chromatography and sedimentation velocity was an inactive monomer with 70% of the native secondary structure, according to circular dichroism results. Moreover, the instability exhibited by the dimer in the presence of the denaturant was due to its dissociation, probably by weakening of the interface interactions between MATα1 monomers. In fact, we were able to calculate that approximately 50% of the global stabilization energy of MAT III was due to subunit association, whereas only 25% derived from the interactions stabilizing the intermediate state. MAT I unfolding was also studied taking advantage of the differences in fluorescence intensity exhibited by tetramers and dimers upon 8-anilinonaphtalene-1-sulfonic acid (ANS) binding. The results obtained suggested that around 65% of the global free energy derives from dimer dissociation, thus supporting a lower stability for dimer-dimer interactions (62). On the other hand, thermal denaturation of MAT I and MAT III was shown to be irreversible, regardless of the techniques used to follow the process. Only one transition was observed for both tetramer and dimer denaturation, hence suggesting that stability of

MAT I is highly dependent on that of the dimer (67). Using two-dimensional infrared spectroscopy and the structural data it was possible to ascribe the earliest changes to the most exposed elements, α-helices and β-turns.

The high level of sequence and structural conservation among α-subunits suggested no large differences in folding pathways for MATα1 and MATα2 monomers, although association steps may diverge due to the incorporation of MATβ into MAT II hetero-oligomers. However, this initial hypothesis was demonstrated to be partially wrong when refolding of MATα2 was performed. In fact, this subunit followed a similar pathway to produce MATα2 dimers, and only in a latter step association of MATβ takes place (46). Data regarding folding pathways *in vivo* remain very limited, although *E. coli* MAT was identified several years ago among the substrates for the chaperonin GroEL (68).

All this information regarding folding and structure could be of help to understand the impact of the different *MAT1A* mutations detected in patients with isolated persistent hypermethioninemia, and in fact we performed this exercise in 2011 (69). Most of these patients are asymptomatic, although demyelination and other central nervous system manifestations have been described in several cases (70,71). The number of patients is still small, but newborn screenings in search for *CBS* defects are aiding in the identification of hypermethioninemias due to *MAT1A* mutations. Most mutations follow a recessive autosomal inheritance trait, although a dominant pattern has been described for the transition leading to the R264H protein mutant (72). The presence of early stop codons leading to shorter MATα1 subunits has been reported, as well as the possibility of longer chains due to mutations in the stop codon or splice-donor sites (70,71,73). Analysis of some of these mutant proteins has been carried out upon overexpression in *E. coli* and/or COS cells and their effects on activity evaluated.

The early stop codons identified to date lead to proteins lacking from part (386X) or most (350X) of the C-terminal domain, to others in which even information for a complete domain is absent (92X and 185X). Additional early stop codons (351X) result not only in the loss of half of the C-terminal domain, but also in a change of sequence that affects a portion of the central and the remaining C-terminal domains. In contrast, longer subunits resulting from the stop codon mutation (X396Y-464X) present with the whole wild type sequence, and hence the information for the whole N- and central domains plus and extended C-terminal domain. Longer monomers due to splice-donor mutations on exon III, will result in proteins whose sequence is drastically altered, and hence, their folding and structure. If not eliminated by proteolysis, the shortest MATα1 subunits are expected to result in a complete change in the folding pattern, precluding association. However, those mutants in which the sequences for the N- and central domains remain intact, and even part of the C-terminal domain is preserved, may achieve an intermediate state that allows association (69).

This is the case for mutations leading to MATα1 subunits of approximately 350 residues, which were the first identified in patients with demyelination. In fact, Hazelwood *et al*. proposed that this type of subunits might associate with MATα2 monomers, leading to an inactive hetero-oligomer (74). Although plausible, given the high level of sequence identity between MATα1 and MATα2 subunits, the availability of new structural data showed that there are small differences in the orientation of the monomers. Moreover, these mutants ~350 residues long lack the C-terminal domain sequence involved the in monomer-monomer interface, including the β-sheet that participates in dimer association. These divergences together will impose a rearrangement to avoid exposure of amino acids that are hidden in the wild type MATα1, and hence reducing the probability of association with MATα2 (69). Once

again, additional experiments are needed for verification of the folding pathways followed by the mutants, their putative ability to hetero-oligomerize with wild type subunits, etc.

4.4 Oxidative stress induced by copper accumulation impairs SAM synthesis in early stages of Wilson disease.

Several models of liver disease that induce oxidative stress by different mechanisms have been explored along the years regarding the alterations induced in SAM synthesis. Just to name some, carbon tetrachloride intoxications (75,76), treatments with buthionine sulfoximine (BSO) (42), Long Evans Cinnamon (LEC) rats (20-22), etc. all concur with a reduction in MAT activity and SAM levels. Now that the role of cysteines in MAT I and MAT III is known this is no longer a surprise, but since most of these models were explored earlier there was a need to revisit certain aspects. Among them, the case of the Wilson disease model provided by LEC rats remained underexplored, probably because of the rare condition of this pathology. The available data referred only to activity parameters and were determined in an advanced stage of the disease, where hepatitis was already present. Therefore, we decided to explore early pathological stages to get insight into the copper effects on SAM synthesis. For this purpose, we examined 9-week old LEC rats, which already presented with copper deposits in the liver, but no signs of hepatitis (20).

Hepatic copper accumulation at this early step led to changes in expression that were unexpected for MAT genes (20). The *Mat1a*/*Mat2a* expression switch was already detected in LEC rats at this age, but *Mat2b* expression was dramatically reduced, hence following an opposite pattern to that classically described in liver disease. These effects on *Mat2b* expression were prevented by neocuproine, a chelator of Cu (I) (Figure 4). Reductions in SAH concentrations were larger than effects on SAM levels, probably

due to the effects of copper on SAHH (47). Activity measurements carried out under conditions that allowed distinction between the three MAT isoenzymes indicated a decrease in MAT III activity, together with an increase in the combined activity of MAT I and MAT II. These effects on activity correlated with an enhancement in the MAT I content together with a reduction in that of MAT III, according to analytical gel filtration chromatography (AGFC) and dot blot detection of MATα1 (20).

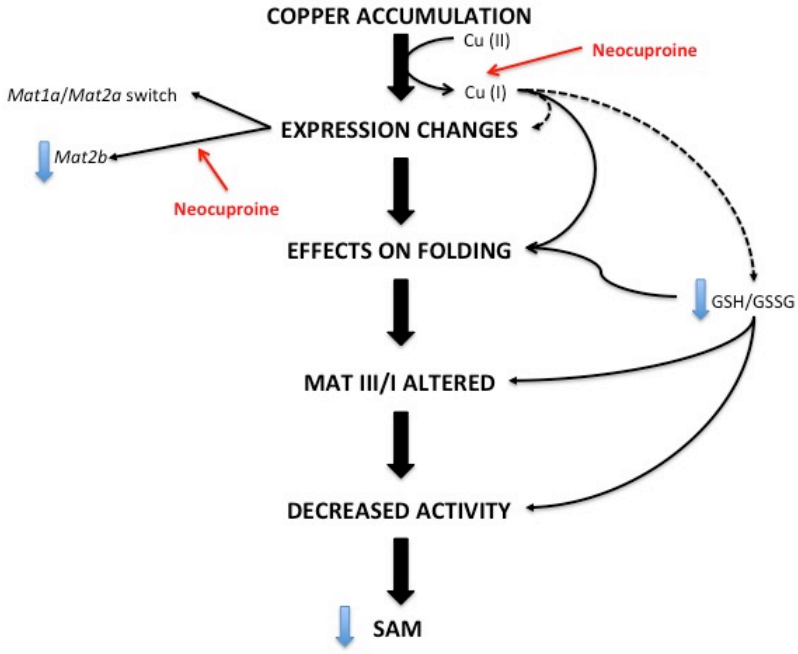

Figure 4. Summary of the effects detected on S-adenosylmethionine synthesis in LEC rats. The different levels at which copper accumulation alters SAM synthesis are shown, as well as the modifications prevented by neocuproine administration.

Further analyses of copper effects on MAT proteins were carried out *in vitro* taking advantage of the refolding protocol. These experiments showed effects on folding of MATα1 and MATα2, the presence of copper reducing the amount of soluble protein obtained, but having no effect in the association state attained. Moreover, addition of copper to correctly folded MATs had a direct effect on their activity (20). MATα2 dimers were more susceptible to inhibition than MAT I and MAT III under reducing conditions, whereas this pattern was opposite when reducing conditions were

milder. This effect of copper on activity correlated with the generation of high-Mr aggregates, further supporting its role in the folding of MATs. In this case, the residues affected by oxidative stress remain unknown, and hence further studies should be carried out to understand the mechanism of copper effects on these proteins.

4.5 The affinity between MAT II subunits is increased by $NADP^+$ binding.

Several years latter crystal structures of human MATα2 homo-oligomers became available, monomers showing the same organization than MATα1, including three domains and a similar pattern for dimerization. Following this achievement structures of MATβ homo-oligomers were solved and the presence of a Rossman-fold including $NADP^+$ demonstrated (77). However, no explanation of the cofactor's role in the protein was provided. Therefore, we adapted the refolding protocol of the laboratory for human MATα2 production, and improved the methods for recombinant human MATβ production and purification (46). Using isothermal titration calorimetry we were able to determine the affinity parameters for $NADP^+$ binding to MATβ, to demonstrate that the cofactor increases the affinity of MATβ for MATα2 subunits, and finally that MAT II is a hetero-trimer composed by a MATα2 dimer and a single MATβ subunit (46).

The structural data available also allowed the preparation of models for MAT II structure, the best approach suggesting that the interaction between the MATα2 dimer and MATβ not only involves the $NADP^+$ binding site, but takes place close to the protein active site. The role of MATβ residues postulated to bind the cofactor was proven by site-directed mutagenesis, whereas additional mutants lacking part of the N-terminal (similar to the V2 splicing form) showed altered kinetic properties upon their association with MATα2 dimers (46). Several of our findings were confirmed by Murray et al. in 2014, including the stoichiometry between MATα2 and MATβ

subunits (2:1), and the fact that the N-terminal end of MATβ regulates the activity of MAT II (46,78). However, their crystal structures of MAT II showed a different interaction surface for the subunits, suggesting a role for the C-terminal end of MATβ (in this case the V2 splicing form) and a tunnel at the interface of the MATα2 dimer (78). Again, and in spite of the effort already performed, more structural work is needed to clarify the disparities reported to date.

4.6 MAT1A expression is not restricted to the liver.

Northern blots carried out on samples from animal models or human biopsies showed *MAT1A* expression in liver, together with a switch towards *MAT2A* expression in liver disease (4,13,79,80). Moreover, the *Mat1a*$^{-/-}$ mouse developed severe hepatic pathology with age, most of these mice showing hepatocellular carcinoma (81). In contrast, most patients with hypermethioninemia due to mutations in this same gene either do not develop symptoms or those are neurological, including a few cases showing signs of demyelination by magnetic resonance imaging (MRI), as explained in a previous section (70,71,82). Therefore, in our opinion, there was a need to reexamine the tissue expression profile of *MAT1A*. Using real-time RT-PCR this analysis was performed in rat tissues, where expression was detected in almost all of those examined. Several expression levels were found: 1) high, shown in liver; 2) medium, exhibited by pancreas and lung; and 3) low, found in the rest of the tissues examined (83). This same pattern was further confirmed by western blot using an anti-MATα1 antibody developed in the last years in our laboratory using highly purified preparations of the protein (64,83).

4.7 MATs are cytoplasmic and nuclear proteins.

Localization of MATs to the cytoplasm was a well-established characteristic, based mainly in traditional subcellular fractionation studies and activity measurements.

However, lack of good antibodies precluded confirmation of this statement for a long time. Again, using the new available antibody and immunohistochemistry we were able to detect MATα1 in both the cytoplasm and the nucleus, although nuclear localization was preferred in extrahepatic rat tissues (83). This discovery led to new questions regarding whether there was nuclear MAT activity and which were the isoenzymes present in that subcellular compartment. The answer to these points required the development of improved subcellular fractionation protocols and activity measurements that allowed not only detection of the small amount present in the nucleus, but also, and more important, to guarantee lack of contamination from the cytoplasm. In order to detect nuclear MAT activity, a highly concentrated nuclear extract from a whole rat liver was required, together with 4-fold increased levels of the radioactive tracer and longer reaction times. In parallel, LDH activity was measured to ensure complete elimination of the cytoplasm, and even more critical, the activity measurements (LDH and MAT) were carried out in the supernatant before nuclear protein extraction to ensure that the detected activity was specific of the nuclear compartment. Following this protocol, the specific MAT activity detected in the nucleus was approximately 250-fold lower than in the cytoplasm (83). Regarding the nuclear isoenzymes, AGFC followed by dot-blot detection of MATα1 showed the protein as tetramers and monomers, the amount of MAT I being small (83). Therefore, the low nuclear MAT activity measured correlated with the presence of equivalent levels of MAT I in this compartment.

4.8 Overlapping areas of the C-terminal end are involved in the subcellular distribution of MATα1.

SAM is a small compound that can be transported through the nuclear pore, but this structure limits the size of the proteins that can be translocated from one side to another of the nuclear membrane to near 50 kDa. This is almost the size of MAT α-

subunits and is far below that of MAT I (180 kDa). Thus, understanding how MATα1 is distributed between the nucleus and the cytoplasm was our next objective. Many nuclear proteins present localization signals (NLSs) in their sequence (84), others contain conformational signals that mimic the classical NLSs (85), and in other cases binding to proteins including appropriate signals is preferred (piggy-back)(86). Among NLSs, the best-known or classical signals are mono- and bi-partite sequences rich in basic residues, for whose identification several bioinformatic tools are available. Using this software we were unable to identify a classical NLS in the rat MATα1 sequence, and hence the crystal structure was utilized to determine the existence of exposed clusters of basic residues that could mimic such NLSs. Our analysis revealed that the C-terminal of MATα1 is abundant in basic residues, and those exposed to the surface were selected for site-directed mutagenesis. The subcellular distribution of the mutants was analyzed by confocal microscopy and subcellular fractionation and the combined results allowed the identification of two overlapping areas in the monomer structure that were involved either in nuclear localization or cytoplasmic retention (83).

Subcellular localization was independent of MAT activity, since an inactive mutant prepared on a residue involved in methionine binding (F251D) showed the same distribution than the wild type protein (83). Moreover, deletions at the C-terminal end, similar to those found in some patients showing demyelination (lacking 45 residues), favored nuclear localization of the truncated MATα1 (83). Additionally, it was demonstrated that nuclear localization of MATα1 correlated with increased levels of the trimethylation of lysine 27 in histone 3 (me3K27H3) (83), a well-known epigenetic repression mark. In contrast, other histone methylations examined were increased by overexpression of MATα1, independently of the subcellular distribution observed for the mutants.

In 2011, MATα2 was also found in the nucleus during a proteomic analysis in search for protein-protein interactions of the MafK transcription factor (87). This study showed MATα2 acting as a transcriptional corepressor of MafK on the heme oxygenase 1 (HO-1) gene. Moreover, a role for MATβ and MAT activity in this repression system was described. This manuscript together with an Editorial in the same issue of Molecular Cell (87,88), supported the hypothesis, previously postulated by our group (83), suggesting that MATs are transported to nuclear locations where SAM synthesis is needed, whereas organelles such as the mitochondria use transport systems to obtain the compound from the cytoplasm.

4.9 Nuclear accumulation of MATα1 is induced during acute liver injury.

The results described in the previous section suggested that changes in MATα1 subcellular distribution might be related to disease development. In order to examine this possibility, we choose two rat models of acute liver injury that were previously used for partial characterization of alterations in cytosolic methionine metabolism (89-91). Precisely, D-galactosamine and paracetamol intoxications were known to induce reductions in MAT activity and SAM levels (89,90,92). These changes in the D-galactosamine model were ascribed to an anomalous ratio of the MAT isoenzyme activities towards enhanced MAT III activity (89,90). We were able to reproduce these previous results in the cytosol, reductions in total MAT activity being more modest for paracetamol than for D-galactosamine treatments (92,93). The mechanism of action of both drugs is quite different, D-galactosamine inducing depletion of the uridine pool (94), and thus highlighting the interest of analyzing putative expression changes in that model. After 48 hours of D-galactosamine treatment, changes in the mRNA levels of most genes in the methionine cycle were evident, namely: i) the *Mat1a*/*Mat2a* expression switch, followed by a more modest increase in *Mat2b* expression; ii)

increased mRNA levels for *Ahcy* and *Mtr*; iii) enhanced expression of genes involved in glutathione synthesis; and iv) decreased expression of *Gnmt* and *Bhmt* (93). In general, genes considered "exclusively hepatic" showed lower expression.

Additional analyses carried out showed that the changes induced by D-galactosamine in the cytosolic protein levels of MATα1, BHMT, SAHH and GNMT closely matched the alterations observed in their expression (93). Focusing specifically on MATα1, the protein levels were reduced by ~50%, this decrease correlating with a severe alteration in the isoenzyme pattern according to AGFC profiles, where only a small amount of MAT III was detected (93)(Figure 5). Altogether these results followed the previously published pattern detected by activity measurements in the cytosol (89). Paracetamol intoxication produced a more modest decrease in cytosolic MATα1 protein levels together with a completely different pattern in the MAT isoenzyme profile by AGFC, where an increase in MAT I content was readily observed. Moreover, while the MAT III/I activity ratio increased in the cytosol of paracetamol treated livers, the opposite trend was followed by the protein ratio, thus suggesting a certain degree of inactivation of MAT I in these samples (93).

Analysis of the nuclear effects of the treatments revealed accumulation of MATα1 in this compartment both by D-galactosamine and paracetamol intoxications (93). AGFC revealed increased levels of MAT I and a reduction in those of MATα1 monomers in nuclear fractions of D-galactosamine-treated livers, which correlated with the detection of enhanced nuclear MAT activity (Figure 5). In contrast, paracetamol induced a modest increase in nuclear MAT I levels, but no significant changes in activity were measured. However, in both models enhanced levels of the me3K27H3 repression mark were also detected. This lack of correlation between activity and the histone modification in paracetamol intoxication prompted us to evaluate nuclear SAM

levels. However, the procedures needed to purify nuclear fractions precluded this measurement, given the exchange through the nuclear pore that takes place during the extensive washing steps required for isolation. These measurements will be only possible when appropriate imaging techniques become available.

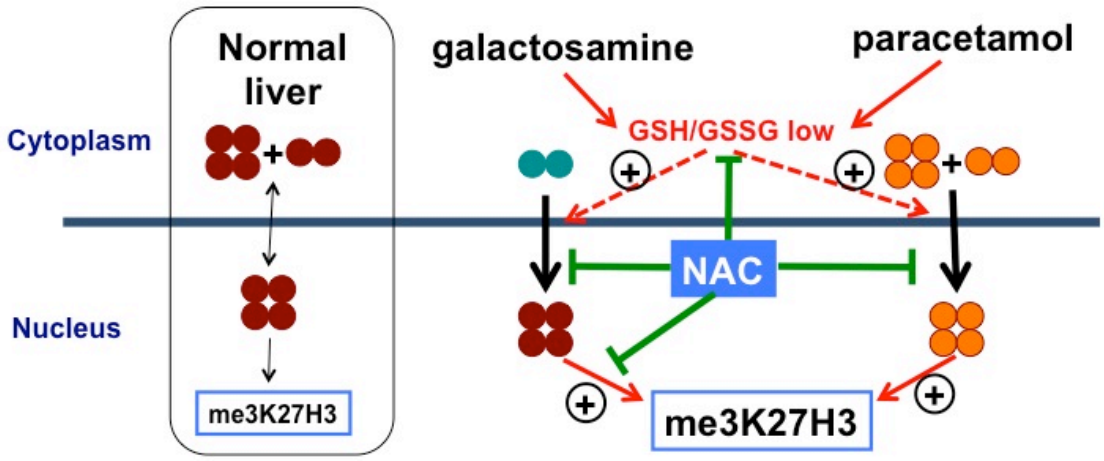

Figure 5. Summary of the effects detected in animal models of acute liver injury. A schematic representation of the hepatic changes induced by acute D-galactosamine and paracetamol intoxications on glutathione levels, MATα1 subcellular localization and a repression signal in liver are depicted. The preventive effects of N-acetylcysteine (NAC) are also shown.

4.10 Changes in the ratio between reduced and oxidized glutathione forms controls the nucleocytoplasmic distribution of MATα1.

D-galactosamine and paracetamol are known to induce redox stress and to reduce the GSH/GSSG ratio. Although the mechanism by which D-galactosamine exerts this effect seems rather indirect, this is not the case for paracetamol, whose detoxification requires GSH consumption, thus leading to altered GSH/GSSG ratios (94). As mentioned previously, my own work demonstrated in 1992 that MAT I and MAT III activities were modulated by the GSH/GSSG ratios and, at extreme concentrations of GSSG, even the association state was modified (41). Therefore, we decided to explore whether glutathione ratios exerted an additional role in the control of MATα1 subcellular distribution using a cellular system, which allowed multiple

combinations of drugs. Available hepatic cell lines include hepatomas and iPS-hepatocytes with low or no *MAT1A* expression (79,95), and primary cultures of hepatocytes rapidly loose expression of this gene (40,96). Therefore, transfection was needed to explore MATα1 behavior in any of these systems, among which we chose the hepatoma H35 cell line for overexpression of tagged-MATα1 (93). D-galactosamine treatment in this system reproduced the changes detected in the rat model: i) reduction in the GSH/GSSG ratio; ii) decreased cytosolic levels of MATα1; iii) accumulation of MATα1 in the nucleus; and iv) increase of the me3K27H3 signal. Agents that serve as sources of cysteine for glutathione synthesis, N-acetylcysteine (NAC) and SAM, were then combined with D-galactosamine treatment and their preventive effects evaluated (Figure 5). Among them, only NAC was effective in preventing all the changes induced by the drug (93).

In this same cellular system the effects induced by paracetamol in the rat model were also reproduced, namely: i) GSH depletion with the concomitant decrease in the GSH/GSSG ratio; ii) nuclear accumulation of MATα1; and iii) increased levels of the me3K27H3 repression mark. However, in this case the combination of paracetamol with NAC only prevented effects on glutathione levels and MATα1 localization, whereas me3K27H3 levels remained elevated (93). Further confirmation of the role of GSH/GSSG ratio in the regulation of MATα1 subcellular distribution was obtained using BSO, a compound that inhibits glutathione synthesis. Again, BSO treatment reduced the GSH/GSSG ratio and nuclear MATα1 accumulation was induced in the cells, these effects being prevented by addition of glutathione ethyl ester (93). Differences between D-galactosamine and paracetamol intoxications may relay not only in their effects on glutathione levels, but also in the ability of the paracetamol intermediate NAPQI for protein modification. Such modification involves again

sulfhydryl groups, which are essential for MAT I and MAT III function, and hence additional studies are required to address this aspect.

5. INTEGRATED MODEL OF REDOX REGULATION FOR MATs AND FUTURE RESEARCH

Altogether the available data concerning redox regulation of MATs expand from expression to subcellular localization and were summarized in a hypothesis recently proposed by our group (97) (Figure 6). This new theory expands a previous proposal that we made back in 1992 (98), by adding the accumulated knowledge obtained in these years. Since the purpose of this review is not to revisit these hypotheses, only a few data will be highlighted.

Most efforts in the last decades have been concentrated in understanding the regulation of *MAT* expression, rendering information from transcription factors acting on the promoter (i.e. NFκB), the different gene methylation levels according to the liver state, stabilization of the mRNAs (i.e. AUF1 and HuR binding), etc. Our own work has been concentrated however in the proteins themselves, our results concerning from the structure (i.e. intrasubunit disulfide bond), to cofactor regulation (i.e. $NADP^+$ binding) and, more recently, subcellular distribution of MATα1 and its relationship with disease. Briefly, the proposal indicates that the high SAM levels found in normal liver depend on: 1) low methylation levels of the *MAT1A* promoter allowing gene expression; 2) high *MAT2A* promoter methylation levels, together with decreased *MAT2A* mRNA stability by binding of meHuR; 3) the normal ratios of GSH/GSSG preserving high MAT I/III activity in the cytosol and keeping MATα1 transport to the nucleus at low ratios, therefore maintaining adequate methylation levels of histones and DNA. In contrast, the low SAM levels found in damaged liver are due to the combination of: 1) an inversion

of the promoter methylation levels, which become high for *MAT1A* and low for *MAT2A* promoters; 2) enhanced *MAT2A* and *MAT2B* transcription by the action of redox regulated transcription factors; 3) destabilization of *MAT1A* mRNA by AUF binding, together with stabilization of *MAT2A* transcripts by HuR binding; 4) increased oxidative stress leading to higher levels of $NADP^+$ and reduced GSH/GSSG ratios, which together favor cytosolic MAT II accumulation, the low V_{max} MAT; and 5) reduced GSH/GSSG ratios induce nuclear accumulation of MATα1 and MAT I providing higher nuclear SAM concentrations for epigenetic mechanisms.

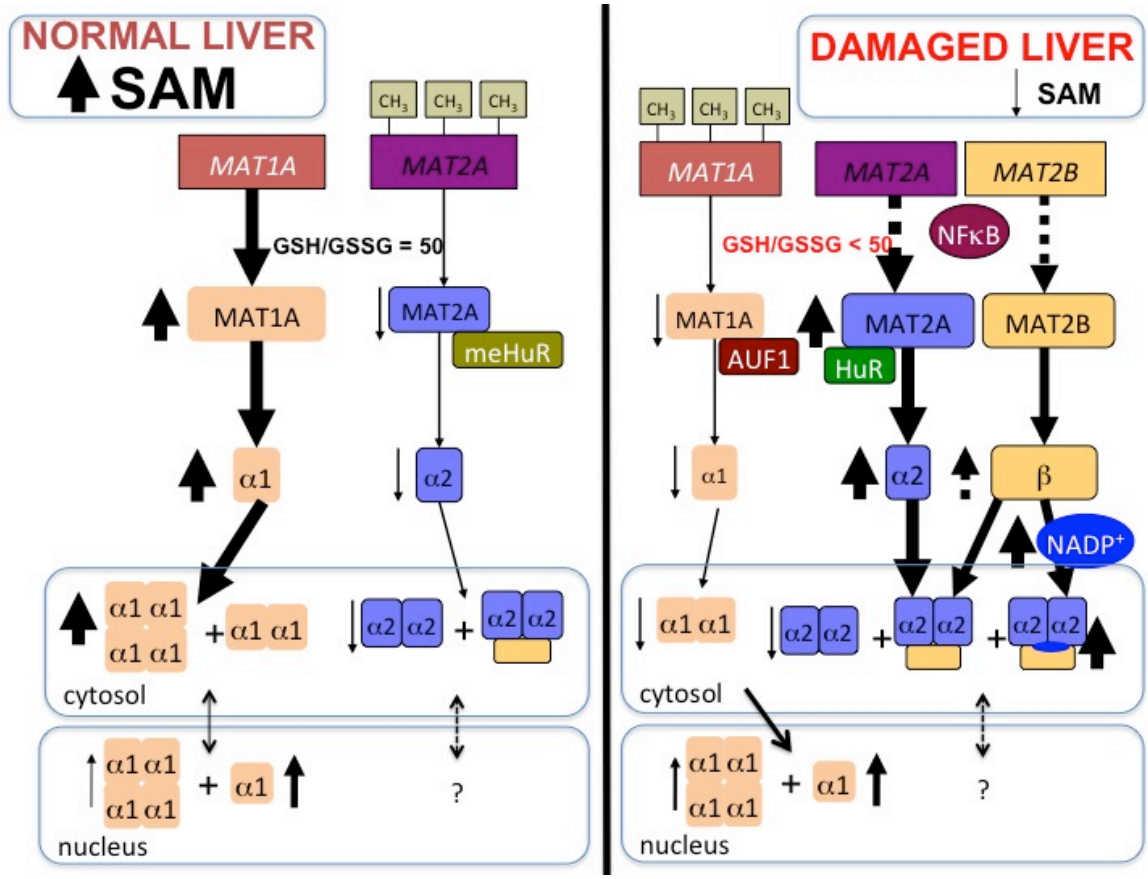

Figure 6. Actualized version of the model proposed for redox regulation of MATs. The proposal includes the most significant data regarding normal liver (left) and damaged liver (right). Mean GSH/GSSG ratios in each situation are included, together with the changes detected in expression due to gene methylation or stabilization/destabilization of the mRNAs. Effects on the proteins include those derived from increased levels of the cofactors, altered association patterns and subcellular distribution rendering finally, high or low SAM levels in normal and damaged livers respectively.

Redox regulation is probably the best-known mechanism controlling MAT function in disease states, but still questions regarding how copper exerts its effects on these enzymes remain unsolved. Additionally, the role of other actors in redox stress, such as NAD^+/NADH or $NADP^+$/NADPH ratios, should be explored in the whole methionine cycle and special attention should be paid on subcellular distribution. The number of enzymes of this pathway found in the nucleus is increasing (SAHH, GNMT, MATs) and their role in this compartment is underexplored. Possibilities such as moonlighting activities cannot be forgotten, neither the protein-protein interactions in which they might be involved.

6. CONCLUSION

In the last 20 years our group has developed protocols and tools for the study of MATs and the methionine cycle and, as a result of this effort, new data have been obtained both *in vitro* and *in vivo*. Altogether, these results have shown the existence of new levels of regulation for MATs that might be key for understanding their role in disease, but which could also be considered as putative targets for the development of new treatments. Moreover, some aspects uncovered during our work might be useful as biomarkers of disease, provided the adequate tissue samples and tools are available. In the near future, work in this field will require a larger effort on the solution of structures, the role of post-translational modifications, the identification of protein-protein interactions and development of imaging techniques able to evaluate subcellular levels of metabolites. Null mice models are valuable tools for the development of the field, but certain aspects concerning the proteins themselves cannot be analyzed in animals that lack their expression. Subcellular localization and its relationship with pathology lay within this category.

7. ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

SAM, S-adenosylmethionine

ADA, adenosine deaminase

ADK, adenosine kinase

AGFC analytical gel filtration chromatography

AMPK, AMP kinase

APAP, paracetamol

BHMT, betaine homocysteine methyltransferase

BHMT2, betaine homocysteine methyltransferase 2

BSO, buthionine sulfoximine

CBS, cystathione β-synthase

CTH, cystathionase

GNMT, glycine N-methyltransferase

GSH, glutathione reduced form

GSSG, glutathione oxidized form

Hcy, homocysteine

LEC, Long Evans Cinnamon

MAT, methionine adenosyltransferase

me3K27H3, trimethylation of lysine 27 in histone 3

MTHF, methyltetrahydrofolate

MTHFR, methylene tetrahydrofolate reductase

MTR, methionine synthase

NAC, N-acetylcysteine